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(54) Title: LACTIC ACID UTILISING BACTERIA AND THEIR THERAPEUTIC USE

(57) Abstract: There is provided a method of isolating novel lactic acid utilising bacteria from human faeces, as well as novel strains so obtained. The use of the novel lactic acid utilising bacteria in therapy, including prophylactic therapy, is described and is of particular relevance for lactic-acidosis, short bowel syndrome and inflammatory bowel disorders such as Crohn's disease and ulcerative colitis. A probiotic comprising the live lactic acid utilising bacteria is also described.

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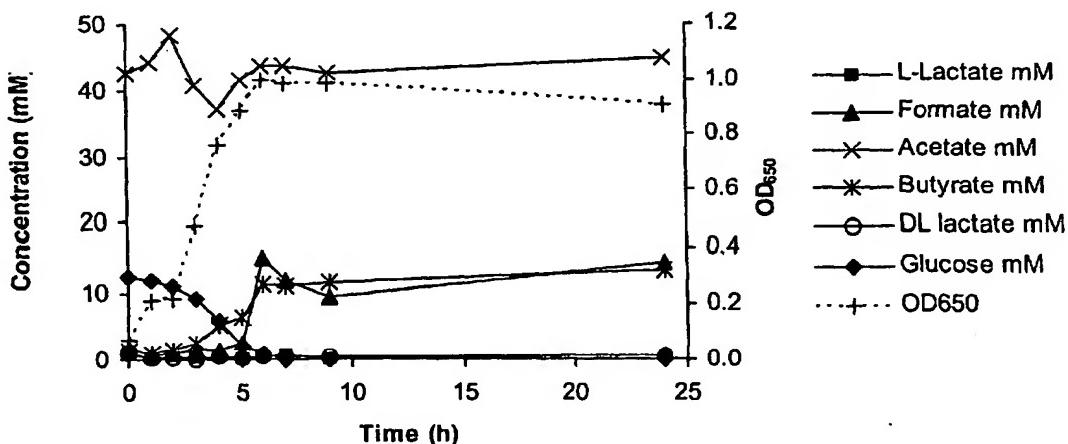
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(54) Title: LACTIC ACID UTILISING BACTERIA AND THEIR THERAPEUTIC USE

SS2/1 (Glucose)



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(57) Abstract: There is provided a method of isolating novel lactic acid utilising bacteria from human faeces, as well as novel strains so obtained. The use of the novel lactic acid utilising bacteria in therapy, including prophylactic therapy, is described and is of particular relevance for lactic-acidosis, short bowel syndrome and inflammatory bowel disorders such as Crohn's disease and ulcerative colitis. A probiotic comprising the live lactic acid utilising bacteria is also described.



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1 Lactic Acid Utilising Bacteria and
2 their Therapeutic Use

3

4 This invention relates to improvements in health and
5 nutrition for both animals and humans following the
6 ingestion of specific bacteria capable of utilising
7 lactic acid.

8

9 Under normal conditions the concentration of lactic acid
10 (lactate) in the mammalian gut is very low despite the
11 fact that many bacterial species, such as lactobacilli,
12 streptococci, enterococci and bifidobacteria that reside
13 in the intestine produce this acid in large quantities as
14 a fermentation end product. Lactic acid is also produced
15 by host tissues.

16

17 It has been hypothesised that the accumulation of lactic
18 acid is normally prevented by the ability of certain
19 other bacteria that inhabit the gut to consume lactic
20 acid and to use it as a source of energy. The identity
21 of the micro-organisms that are postulated to conduct
22 this metabolic process in the mammalian large intestine
23 has largely not previously been elucidated, Bourriaud et

1 al (2002). Kanauchi et al (1999) revealed that a strain
2 of *Bifidobacterium longum* was co-incubated with a strain
3 of *Eubacterium limosum* on germinated barley feedstuff for
4 three days there was a marked increase in acetate formed
5 and a small increase (less than 3 mM) in butyrate formed
6 when compared to the incubations with *E. limosum* alone.

7

8 In the rumen of cattle and sheep the species *Selenomonas*
9 *ruminantium*, *Veillonella parvula* and *Megasphaera elsdenii*
10 are regarded as the most numerous utilisers of lactate
11 (Gilmour et al., 1994; Wiryawan and Brooker, 1995). The
12 contribution of *Megasphaera elsdenii* appears to be
13 particularly significant in the rumen, based on the high
14 proportion of carbon flow from lactic acid to propionic
15 acid and this species employs the acrylate pathway for
16 this purpose (Counotte et al., 1981). *Megasphaera*
17 *elsdenii* produces a variety of end products including
18 propionate, butyrate, caproate and branched chain fatty
19 acids from lactate - see Ushida et al (2002), Kung and
20 Hession, (1995). This probably reflects the ability of
21 this species to use lactate despite the presence of other
22 carbon sources such as sugars, whereas *Selenomonas* uses
23 lactic acid only in the absence of other energy sources.
24 This has led to interest in the use of *Megasphaera* as a
25 probiotic organism that might be added to animal (Kung
26 and Hession, 1995; Ouwerkerk et al., 2002), or even human
27 diets to prevent the harmful accumulation of lactic acid.
28 In ruminant animals (cattle and sheep) accumulation of
29 lactic acid occurs when a large amount of readily
30 fermentable substrate (such as starch and sugars) enters
31 the rumen. Rapid fermentation, particularly by organisms
32 such as *Streptococcus bovis*, drives down the pH, creating

1 more favourable conditions for the proliferation of
2 lactic acid producing bacteria such as lactobacilli, and
3 *S. bovis* itself. Normal populations of bacteria capable
4 of utilising lactate (lactate utilisers) are unable to
5 cope with the greatly increased production of lactic
6 acid. Unaided, lactic acid may accumulate to levels that
7 can cause acute toxicity, laminitis and death (Nocek,
8 1997; Russell and Rychlik, 2001).

9

10 Similar events occurring in the large intestine can also
11 cause severe digestive and health problems in other
12 animals, for example in the horse where high lactate
13 levels and colic can result from feeding certain diets.

14

15 In humans lactic acid accumulation is associated with
16 surgical removal of portions of the small and large
17 intestine, and with gut disorders such as ulcerative
18 colitis and short bowel syndrome (Day and Abbott, 1999).
19 High concentrations of lactic acid in the bloodstream can
20 cause toxicity (Hove et al., 1994), including
21 neurological symptoms (Chan et al., 1994). Much of this
22 lactic acid is assumed to derive from bacterial
23 fermentation, particularly by bifidobacteria and by
24 lactobacilli and enterococci. Lactic acid can also be
25 produced by host tissues, but the relative contributions
26 of bacterial and host sources are at present unclear.

27

28 Conversely, the formation of other acid products, in
29 particular butyric acid (butyrate), is considered to be
30 beneficial as butyric acid provides a preferred energy
31 source for the cells lining the large intestine and has
32 anti-inflammatory effects (Inan et al., 2001, Pryde et

1 al., 2002). Butyrate also helps to protect against
2 colorectal cancer and colitis (Archer et al., 1998;
3 Csordas, 1996).

4

5 We have now established a method of isolating novel
6 bacteria that are remarkably active in consuming lactic
7 acid. The bacteria have been isolated from human faeces.
8 Preferably the method allows isolation of bacteria which
9 convert the lactic acid to butyric acid. According to
10 this method several new bacteria that are remarkably
11 active in converting lactic acid to butyric acid have
12 been isolated.

13

14 One group of these bacteria is from the newly described
15 genus *Anaerostipes caccae* (Schwiertz et al., 2002).
16 Although some main characteristics of *A. caccae* are
17 described in this publication, its ability to use lactate
18 was not reported and has only recently been recognised as
19 described herein.

20

21 The invention relates to a method for selecting a strain
22 of lactic acid-utilising bacteria, which method comprises
23 the steps of:

- 24 a) providing (for example isolating) a bacterial
25 culture from a human faecal sample;
- 26 b) selecting a single colony of bacteria;
- 27 c) growing said colony in a suitable medium
28 containing lactic acid; and
- 29 d) selecting a strain of bacteria consuming
30 relatively large amounts of lactic acid, all of
31 the above steps being conducted under anaerobic
32 conditions.

1 In the above method, the reference to "relatively large
2 amounts of lactic acid" is defined as meaning the
3 bacteria used at least 10 mM of D, L or DL lactic acid
4 during growth into stationary phase, per 24 hours at 37°C
5 in YCFALG or YCFAL medium.

6

7 Preferably the strain of lactic acid utilising bacteria
8 also produces high level of butyric acid and the method
9 of the invention may therefore comprise an additional
10 step of:

11 e) selecting a strain of bacteria producing
12 relatively large quantities of butyric acid.
13

14 In the above step the reference to "relatively large
15 quantities of butyric acid" is defined as meaning the
16 bacteria produces at least 10 mM of butyric acid during
17 growth into stationary phase, per 24 hours at 37°C in
18 YCFALG or YCFAL medium.

19

20 Preferably the strain of lactic acid utilising bacterium
21 must be capable of converting lactate produced by another
22 gut bacterium from dietary components such as resistant
23 starch.

24

25 Preferably the lactic acid used in step c) is both D- and
26 L- isomers of lactic acid.

27

28 Preferably the suitable medium to grow bacteria is
29 nutritionally rich medium in anaerobic Hungate tubes.

30

31 Preferably the selected strain of bacteria is re-purified
32 using nutritionally rich medium in anaerobic roll tubes.

1 A further aspect of the invention is a bacterial strain
2 that produces butyric acid as its sole or predominant
3 fermentation product from lactate and which has been
4 isolated according to the method of the invention
5 described above. Such novel bacterial strains include:

6
7 the bacteria *Anaerostipes caccae* strain L1-92 deposited
8 at NCIMB (National Collections of Industrial, Marine and
9 Food Bacteria in Aberdeen, United Kingdom) under No
10 13801^T on 4 November 2002 and at DSM under No 14662 on 4
11 November 2002.

12
13 the *Clostridium indolis* bacterial strain Ss2/1 deposited
14 at NCIMB under No 41156 on 13 February 2003;

15
16 the bacteria strain SM 6/1 of *Eubacterium hallii*
17 deposited at NCIMB under No. 41155 on 13 February 2003.

18
19 Another aspect of the invention is a strain of bacteria
20 having a 16S rRNA gene sequence which has at least 95%
21 homology to one of the sequences shown in Figure 1,
22 preferably 97% homology (ie. differs at less than 3% of
23 residues out of approximately 1400 from one of the
24 sequences shown in Figure 1).

25
26 Another aspect of the invention is the use of at least
27 one of the above-mentioned bacterial strains in a
28 medicament or foodstuff.

29
30 Another aspect of the invention is a method to promote
31 butyric acid formation in the intestine of a mammal, said
32 method comprising the administration of a therapeutically

1 effective dose of at least one of the above described
2 strains of live butyric acid producing bacteria. The
3 bacterial strain may be administered by means of a
4 foodstuff or suppository or any other suitable method.
5

6 Another aspect of the invention is a method for treating
7 diseases associated with a high dosage of lactic acid
8 such as lactic-acidosis, short bowel syndrome and
9 inflammatory bowel disease, including ulcerative colitis
10 and Crohn's disease, which method comprises the
11 administration of a therapeutically effective dose of
12 *Anaerostipes caccae* or at least one above-mentioned
13 strains of live lactic acid utilising bacteria.

14 Advantageously the strain selected may also produce a
15 high level of butyric acid.

16
17 Further, another aspect of the invention is a
18 prophylactic method to reduce the incidence or severity
19 of colorectal cancer or colitis in mammals caused in part
20 by high lactic acid and low butyric acid concentrations,
21 which method comprises the administration of a
22 therapeutically effective dose of at least one above
23 identified strains of live lactic acid utilising bacteria
24 and/or butyric acid producing bacteria mentioned above or
25 of *Anaerostipes caccae*.

26
27 Another aspect of the invention is the use of live
28 *Anaerostipes caccae* or at least one of the above
29 mentioned lactic acid utilising bacteria as a medicament.
30 Advantageously the strain chosen may produce butyric acid
31 as its sole or predominant fermentation product from
32 lactate. Preferably the bacteria are used in the

1 treatment of diseases associated with high levels of
2 lactic acid such as lactic acidosis, short bowel syndrome
3 and inflammatory bowel disease including ulcerative
4 colitis and Crohn's disease.

5

6 According to another aspect of the invention at least one
7 lactate-utilising strain of bacteria as mentioned above
8 or *Anaerostipes caccae* are used in combination with
9 lactic acid producing bacteria including those such as
10 *Lactobacillus* spp. and *Bifidobacterium* spp. or other
11 additives or growth enhancing supplement currently used
12 as probiotics.

13

14 The combination of strains would potentially enhance the
15 health-promoting benefits of the lactic acid bacterium by
16 converting its fermentation products (lactic acid alone
17 or lactic acid plus acetic acid) into butyrate. Indeed
18 it is possible that certain health-promoting properties
19 currently ascribed to lactic acid bacteria might actually
20 be due to stimulation of other species such as lactate-
21 consumers *in vivo*, particularly where probiotic
22 approaches (see below) are used to boost native
23 populations in the gut. Furthermore the presence of the
24 lactic acid producing bacteria in a combined inoculum
25 could help to protect the lactate consumer against oxygen
26 prior to ingestion.

27

28 The growth and activity of the novel bacteria may be
29 promoted by means of providing certain growth
30 requirements, required for optimal growth and enzyme
31 expression to the bacteria, present in the animal or
32 human gastrointestinal tract. These bacterial growth

1 enhancing nutrients are often referred to as prebiotics
2 or symbiotics.

3 Thus the invention provides methods to promote the growth
4 and enzyme expression of the micro-organism and hence
5 removal of lactate and production of butyrate *in vivo*,
6 for example, via a prebiotic or symbiotic approach
7 (Collins and Gibson, 1999).

8

9 Another aspect of the invention is a method for treating
10 acidosis and colic in animals, particularly in ruminants
11 and horses or other farm animals, by administration of a
12 therapeutically effective dose of *Anaerostipes caccae* or
13 at least one of the lactate utilising bacteria mentioned
14 above. Advantageously the bacteria can be administrated
15 as feed additives.

16

17 For the use, prevention or treatment of conditions
18 described herein, the bacteria or prebiotic(s) or
19 symbiotic(s) are preferentially delivered to the site of
20 action in the gastro-intestinal tract by oral or rectal
21 administration in any appropriate formulae or carrier or
22 excipient or diluent or stabiliser. Such modes of
23 delivery may be of any formulation included but not
24 limited to solid formulations such as tablets or
25 capsules; liquid solutions such as yoghurts or drinks or
26 suspensions. Ideally, the delivery mechanism delivers
27 the bacteria or prebiotic or symbiotic without harm
28 through the acid environment of the stomach and through
29 the rumen to the site of action within the gastro-
30 intestinal tract.

31

1 Another aspect of the invention is the use of at least
2 one bacterial strain mentioned above or *Anaerostipes*
3 *caccae* in a method to produce butyric acid from lactate
4 and acetate. The method includes the fermentation of the
5 above described microorganism selected for both their
6 lactic acid utilising and butyric acid producing
7 abilities in a medium rich in lactate and acetate. The
8 method can be used in industrial processes for the
9 production of butyrate on a large scale.

10

11 **Brief description of the Figure**

12

13 **Figure 1:** Sequence information of 16S rRNA for five
14 lactic acid utilising strains.

15

16 **Figure 2:** Co-culture experiment. Concentration of SCFA
17 are shown after 24 hours growth in YCFA medium with 0.2%
18 starch as energy source (values for acetate, initially
19 present in the medium, are shown on a 10 fold reduced
20 scale). Butyrate production by *A. caccae* L1-92, and by
21 *E. hallii* L2-7 and SM 6/1, is stimulated by co-culture
22 with *B. adoloscentis* L2-32, while L-lactate disappears
23 from the co-cultures.

24

25 **Figure 3:** SCFA formation and lactate utilisation for new
26 and existing isolates. Acids produced or consumed during
27 anaerobic growth are shown for strains incubated for 24
28 hours: a) YCFA medium containing 35mM DL lactate (YCFAL);
29 b) YCFA medium containing 10mM glucose and 35mM DL
30 lactate (YCFALG); c) YCFA medium with no addition.
31 Carbon recoveries (%) for growth on lactate, and lactate
32 plus glucose, respectively, were as follows: SM 6/1

1 (94.6, 76.4); SL 6/1/1 (100.2, 78.7); L1-92 (96.2, 97.9);
2 SS2/1 (92.1, 90.1); SSC/2 (104.4, 96.9); SR1/1 (103,
3 93.8). This suggests that there may be unidentified
4 fermentation products in the case of SM 6/1, SL 6/1/1 and
5 SS3/4 when grown on glucose plus lactate.

6

7 **Figure 4:** Time course of SCFA formation and growth in
8 batch culture of *E. hallii* L2-7 on media containing DL
9 lactate, glucose, or DL lactate plus glucose.

10

11 **Figure 5:** Time course of SCFA formation and growth in
12 batch culture of strain SS2/1 on media containing DL
13 lactate, glucose, or DL lactate plus glucose.

14

15 DETAILED DESCRIPTION

16

17 The experimental work performed shows the following:

- 18 1. Certain human colonic anaerobic bacteria, including
19 A. caccae strains, are strong and efficient
20 utilisers of lactic acid.
- 21 2. Certain human colonic anaerobic bacteria, including
22 A. caccae strains, are strong and efficient
23 producers of butyric acid.
- 24 3. Certain human colonic anaerobic bacteria, including
25 A. caccae strains, convert lactic acid to butyric
26 acid.

27

28

29

30

31

32

1 **Example 1: Isolation and characterisation of bacteria**

2

3 A faecal sample was obtained from a healthy adult female
4 volunteer that had not received antibiotics in the
5 previous 6 months. Whole stools were collected, and 1g
6 was mixed in 9ml anaerobic M2 diluent. Decimal serial
7 anaerobic dilutions were prepared and 0.5ml inoculated
8 into roll tubes by the Hungate technique, under 100% CO₂
9 (Byrant, 1972).

10

11 Bacterial strains were isolated by selection as single
12 colonies from the nutritionally rich medium in anaerobic
13 roll tubes as described by Barcenilla et al. (2000). The
14 isolates were grown in M2GSC broth and the fermentation
15 end products determined. Butyrate producing bacteria
16 were re-purified using roll tubes as described above.
17 Strains L1-92, S D8/3, S D7/11, A2-165, A2-181, A2-183,
18 L2-50 and L2-7 were all isolated using this medium.
19 Omitting rumen fluid and/or replacing the sugars with one
20 additional carbon source such as DL lactate increased the
21 selectivity of the roll tube medium and this medium was
22 used to isolate strain S D6 1L/1. Strains G 2M/1 and SM
23 6/1 were isolated from medium where DL-lactate was
24 replaced with mannitol (0.5%). Separately, non-rumen
25 fluid based media routinely used for isolating
26 Selenomonas sp., namely Ss and Sr medium (Atlas, 1997)
27 was used to isolate other strains. Inoculating Sr medium
28 roll tubes with dilutions of faecal samples resulted in
29 the isolation of strain Sr1/1 while the Ss medium
30 resulted in the isolation of strains Ss2/1, Ss3/4 and
31 Ssc/2.

32

1 **Example 2: *A. caccae* and other human colonic bacterial
2 isolates consumes lactic acid and acetic acid and
3 produces butyric acid when grown in rumen fluid**

4

5 Table 1 summarises the fermentation products formed by
6 twelve strains of anaerobic bacteria when grown under
7 100% CO₂ in a rumen fluid-containing medium containing
8 0.5% lactate (M2L) or 0.5% lactate, 0.2% starch, 0.2%
9 cellobiose and 0.2% glucose (M2GSCL) as the energy
10 sources. Ten of these strains were isolated from human
11 faeces as described above in Example 1. Strains 2221 and
12 NCIMB8052 are stock collection isolates not from the
13 human gut and are included for comparison. Table 1
14 demonstrates that three strains, L1-92 (*A. caccae*), SD6
15 1L/1 and SD 6M/1 (both *E. hallii* -related) all consumed
16 large amounts of lactate (>20mM) on both media examined,
17 M2L and M2GSCL, and produced large quantities of butyric
18 acid. *A. caccae* L1-92 in particular consumed large
19 amounts of lactate and produced large amounts of
20 butyrate. Acetate is also consumed by all three strains.
21 The other 9 butyrate producing bacteria tested either
22 consumed relatively small amounts of lactate, or consumed
23 no lactate, on this medium. L-lactate concentrations
24 were determined enzymatically and glucose concentrations
25 were determined by the glucose oxidase method (Trinder,
26 1969). Analyses were conducted in a robotic clinical
27 analyser (Kone analyser, Konelab Corporation, Finland).

Table 1. Comparison of human faecal isolates for the ability to utilise (negative values) or produce (positive values) lactate on a rumen fluid based medium (M2) supplemented with lactate (M2L) and lactate plus glucose, cellobiose and soluble starch (0.2% each) (M2GSC).

Table 1

Strain ID	Closest relative	Medium	Formate	Acetate	Butyrate	Lactate
S D8/3	Adhufec 406*+	M2L	1.15	0.97		-3.94
S D8/3		M2GSCL	21.66	0.77	10.88	6.43
SL 6/1/1	<i>E. hallii</i>	M2L		-19.74	35.48	-32.41
SL 6/1/1		M2GSCL		-9.78	22.58	-21.85
SM 6/1	<i>E. hallii</i>	M2L	0.79	-19.01	31.73	-23.72
SM 6/1		M2GSCL	1.31	-5.06	22.77	-28.42
G 2M/1	HuCA19*	M2L		2.82	7.97	23.66
G 2M/1		M2GSCL		0.01	12.94	9.52
SD7 11/1	ND [#]	M2L		0.51	0.08	7.58
S D7 11/1		M2GSCL	1.85	0.43	4.84	-10.25
2221	But. Fibrisolvens	M2L	1.37	-3.61	1.57	21.57
2221		M2GSCL	19.4	-5.57	18.02	11.75
8052	<i>Ct. acetoxylycium</i>	M2L		-12.42	19.31	-0.87
8052		M2GSCL	0.13	-1.79	18.00	-5.80

Strain ID	Closest relative	Medium	Formate	Acetate	Butyrate	Lactate
A2-165	<i>F. prausnitzii</i>	M2L	1.98	0.62	3.56	2.94
A2-165		M2GSCL	17.47	-6.97	18.38	-5.65
A2-183	<i>Roseburia</i> sp.	M2L		0.86	1.84	10.63
A2-183		M2GSCL	-0.15	-12.70	18.23	5.33
A2-181	<i>Roseburia</i> sp.	M2L	0.58	-0.26	1.75	
A2-181		M2GSCL	0.33	-11.05	18.68	5.22
I2-50	<i>Coprococcus</i> sp.	M2L	1.06	2.32	0.52	0.43
I2-50		M2GSCL	19.37	4.47	7.60	3.41
L1-92	<i>Anaerostipes caccae</i>	M2L		-29.42	37.00	-25.60
L1-92		M2GSCL	0.63	-27.03	44.78	-45.48

* clone library sequence, uncultured (Hold et al., 2002)

+ clone library sequence, uncultured (Suau et al., 1999)

ND not determined

1 Example 3: *A. caccae* and other human colonic bacterial
2 isolates consumes lactic acid and acetic acid and
3 produces butyric acid when grown in rumen fluid free
4 medium

5

6 Table 2a shows the utilisation and production of formate,
7 acetate, butyrate, succinate and lactate, on this
8 occasion performed using the rumen fluid-free medium YCFA
9 (Duncan et al. 2002) containing no added energy source,
10 or with 32 mM lactate (YCFAL) or lactate plus 23 mM
11 glucose (YCFALG) as added energy sources. Separately
12 Table 2b reveals the levels of the two isomers of lactate
13 (D and L) remaining at the end of the incubations and the
14 concentration of glucose metabolised during the
15 incubations. Five additional new lactate-utilising
16 isolates were discovered using the semi-selective medium
17 as described earlier and are included in Tables 2a and
18 2b, although one of these (Ss 3/4) proved to consume a
19 relatively small amount of lactate only on the YCFAL
20 medium (Table 2a). Analysis of the consumption of the D
21 and L isomers reveals that three strains (Ss2/1, Ssc/2
22 and Sr1/1) preferentially consumed D lactate. Partial
23 repression of lactate consumption by glucose was observed
24 on this medium with *A. caccae* L1-92, and almost complete
25 repression for SL 6/1/1 and Ss 3/4. The previously
26 isolated *E. hallii* strain L2-7 (Barcenilla et al., 2000)
27 behaved in a similar manner to SL 6/1/1. The higher
28 glucose concentration in this medium compared with M2GSCL
29 is likely to explain the difference in behaviour of *A.*
30 *caccae* compared with Table 1. The remaining five strains
31 showed no evidence of repression of lactate utilisation
32 in the presence of glucose although it is possible they

1 use the glucose before switching to lactate. Butyrate
2 levels exceeding 30mM were obtained for four strains on
3 YCFALG medium.

4

5 **Results :** The three *E. hallii*-related strains (L-27, SL
6 6/1/1, SM 6/1) and the two *A. caccae* strains (L1-92 and
7 P2) were able to use both the D and L isomers of lactate
8 during growth either on DL lactate or DL lactate plus
9 glucose (Fig. 3). The four remaining new isolates SR1/1,
10 SSC/2, SS2/1 and SS3/4 however showed a strong preference
11 for using D-lactate. In most strains, except SS3/4 and
12 L2-7, some utilisation of lactate was detectable
13 following 24 hours incubation even when glucose was
14 initially present in the medium (Fig. 3).

15

16 **Table 2a.** Fermentation products formed or utilised (U as
17 indicated by minus values) by human gut isolates
18 incubated on yeast extract-casitone-fatty acids medium
19 (YCFA); YCFA supplemented with lactate (YCFAL); and YCFA
20 supplemented with glucose and lactate (YCFALG). The
21 initial concentration of glucose added to the medium was
22 23 mM and 32 mM lactate was added that contained 15.5 mM
23 L-lactate.

24 ^a Strain identity is based on 16S rRNA sequence
25 information (% identical residues with closest relative
26 is shown). See Figure 1 for sequence information.

All strains except 2221 and 8052 (Table 1) were isolated as described in Example 1.

Table 2a

Strain ID	Closest relative ^a	Isolation Medium	Medium	Formate	Acetate P/U	Butyrate	Succin	Lactate P/U
Ss2/1	<i>Cl. indolis</i> (95%)	Selenomonas selective	YCFIA	0.02±0.04	-4.25±4.68	2.24±0.26		0.39±0.03
			YCFAL	0.18±0.02	-12.51±1.27	12.98±0.19		-15.27±2.53
			YCFALG	10.10±1.05	-24.32±1.03	35.69±1.13		-13.95±2.70
Sr 1/1	<i>Ruminococcus obaeum</i>	Selenomonas ruminantium	YCFIA		-5.42±1.77	2.33±0.03	0.36±0.12	
	HucB 12*		YCFAL	0.76±0.19	-13.35±2.27	14.15±0.17		-15.04±0.89
			YCFALG	9.53±2.03	-22.47±1.40	35.77±1.50		-13.71±0.40
SL 6/1/1	<i>E. hallii</i>	M2 + 0.5% lactate	YCFIA		-4.96±3.26	1.42±0.23		
	HucA 15*		YCFAL					
			YCFALG	-18.51±0.96	21.06±1.06			-29.93±0.60
SM 6/1	<i>E. hallii</i> (98%)	M2 + 0.5% mannitol	YCFIA	0.09±0.03	-2.61±2.36	1.42±0.05		-2.43±0.70
			YCFAL	0.21±0.1	-7.20±2.08	6.54±0.43		-6.27±1.27
			YCFALG	20.68±	-10.95±	29.2±		-25.82±
Ss 3/4	<i>Ruminococcus gnavus</i>	Selenomonas selective	YCFIA		4.75±2.20	6.10±0.27	1.09±0.47	
	HucA19*							

Strain ID	Closest relative ^a	Isolation Medium	Medium	Formate	Acetate P/U	Butyrate	Succin	Lactate P/U
Ss 3/4	<i>Ruminococcus</i> <i>gnavus</i> HucA19*	Selenomonas selective	YCF A	4.75±2.20	6.10±0.27			1.09±0.47
			YCF AL					
				6.68±2.09	6.19±0.34			-9.78±2.56
Ssc/2	<i>Cl. indolis</i> (95%)	Selenomonas selective	YCF A	0.54±0.13	5.06±4.28	8.66±0.53		3.86±1.09
			YCF ALG					
				4	2.37±0.09			0.48±0.03
			YCF AL	0.36	-12.12	13.49		
			YCF ALG	10.98±1.27	25.35±2.87	36.10±0.49		-13.78
L1-92	<i>A. caccae</i> (type strain)	M2GSC	YCF A	0.00±0.08	-2.35±2.03	1.99±0.09		-13.34±1.28
			YCF AL	-0.05±0.10	-21.98±2.45	23.35±1.16		-28.92±0.54
			YCF ALG	1.49±0.13	-26.83±0.58	36.81±3.61		-12.01±1.32
L2-7	<i>E. hallii</i>	M2GSC	YCF A	0.02±0.01	-1.58±1.73	0.63±0.03		0.00±0.00
			YCF AL	1.09±1.55	-14.77±0.93	22.58±0.76		-30.47±0.00
			YCF ALG	3.93±3.38	12.78±0.94	5.80±0.97		1.67±0.47

* clone library sequences, uncultured (Hold et al., 2002)

Table 2b. Total lactate (mM) remaining in the tubes at the end of the 24 h incubation period and separately the concentration of the two forms D and L. Total glucose (gluc) metabolised during growth also recorded (mM).

Strain number	Closest relative	Medium	Total lact.	L-lact	D-lact	Gluc used
Ss2/1	<i>Ct. indolis</i> (95%)	YCFA	0.84±0.02			
		YCFAL	17.08±2.53	16.07±0.40	1.01±2.15	
		YCFALG	18.40±2.70	15.90±1.06	2.50±3.30	22.1±0.0
Sr 1/1	Huc B12*	YCFA	0.81±0.12			
		YCFAL	17.31±0.89	15.05±0.34	2.26±0.68	
		YCFALG	18.64±0.40	16.37±0.79	2.27±0.71	22.0±0.2
SL 6/1/1	<i>E. hallii</i>	YCFA	0.00±0.00			
	Huc A15*					
		YCFAL	2.42±0.60	0.21±0.10	2.21±0.51	
		YCFALG	29.92±0.07	10.65±0.69	19.27±0.79	22.1±0.1
SM 6/1	<i>E. hallii</i> (98%)	YCFA	0.00±0.00			
		YCFAL	26.08±1.27	9.94±0.50	16.14±1.06	
		YCFALG	6.57±0.16	4.02±2.26	2.55±2.32	22.1±0.1
Ss 3/4	HucA19* (new species to be named)	YCFA	1.54±0.47			
		YCFAL	22.58±2.55	16.56±0.12	6.02±2.65	

Strain number	Closest relative	Medium	Total lact.	L-lact	D-lact	Gluc used
Ss 3/4	HucA19* (new species to be named)	YCFALG	6.57±0.16	4.02±2.26	2.55±2.32	22.1±0.1
		YCFA	1.54±0.47			
Ssc/2	<i>A. cacciae</i> (L1-92)	YCFAL	22.58±2.55	16.56±0.12	6.02±2.65	
		YCFALG	36.21±1.09	16.95±0.87	19.26±1.91	16.6±0.6
L1-92	<i>A. cacciae</i> (type strain)	YCFA	0.96±0.08			
		YCFAL	22.39±6.63	15.40±0.78	6.99±6.10	
L2-7	<i>E. hallii</i>	YCFALG	19.01±1.28	15.08±0.93	3.93±0.68	22.2±0.0
		YCFA	0.0±0.0			
		YCFAL	3.43±0.54	1.84±0.85	1.59±0.87	
		YCFALG	20.34±1.32	8.63±0.72	11.71±2.01	
		YCFA	0.00±0.00			
		YCFAL	0.00±0.00			
		YCFALG	31.93±0.47	15.43±0.12	16.50±0.30	11.99±0.71

- clone library sequence, uncultured (Hold et al., 2002)

1 Example 4 : 16S rRNA sequencing of new isolates and
2 phylogenetic relationships

3

4 Cell pellets from 1ml cultures grown on M2GSC medium (24
5 h) that were resuspended in 50µl of sterile d.H₂O served
6 as templates for PCR reactions (0.5µl per 50µl of PCR
7 reaction). 16S rRNA sequences were amplified with a
8 universal primer set, corresponding to positions 8-27
9 (27f, forward primer, AGAGTTGATCMTGGCTCAG) and 1491-1511
10 (rP2, reverse primer ACGGCTACCTTGTACGACTT) of the
11 *Escherichia coli* numbering system (Brosius, 1978;
12 Weisberg, 1991) with a MgCl₂ concentration of 1.5 mM. PCR
13 amplifications were performed using the following
14 conditions: initial denaturation (5 min at 94°C), then 30
15 cycles of denaturation (30 s at 94°C), annealing (30 s at
16 51°C), and elongation (2 min at 72°C), and a final
17 extension (10 min at 72°C). The amplified PCR products
18 were purified using QIA quick columns (Qiagen GmbH,
19 Germany) according to manufacturer's instructions and
20 directly sequenced using a capillary sequencer (Beckman)
21 with primers 27f, rP2, 519f(CAGCMGCCGCGTAATWC) and 519r
22 (GWATTACCGCGGCKGCTG) (corresponding to positions 518-535
23 of the *E. coli* numbering system) and 926f
24 (AAACTCAAAGAATTGACGG) and 926r (CCGTCAATTCTTTAGTTT)
25 corresponding to positions 906-925). Two independent PCR
26 products were sequenced per strain.

27

28 Similarity of the 16S rRNA sequences (minimum 1444 bases)
29 of the isolates with other organisms was compared with
30 all sequence data in GenBank using the BLAST algorithm
31 (Altschul, 1990).

32

1 **Example 5 : Co-culture of lactate utilisers with**
2 ***Bifidobacterium adolescentis***

3
4 Three lactate utilising strains, *Anaerostipes caccae* L1-
5 92 and two strains of *Eubacterium hallii* (SM 6/1 and L2-
6 7) were incubated alone and in co-culture with *B.*
7 *adolescentis* L2-32 on YCFA medium modified to contain
8 reduced casitone (0.1%) and 0.2% soluble starch as an
9 added energy source. The inoculated tubes were incubated
10 for 24 h at 37°C. *B. adolescentis* L2-32 was enumerated
11 on Mann Ragosa Sharpe (MRS) medium containing 2.0% agar
12 with a final concentration of 0.5% propionate and the
13 three butyrate producing strains, were enumerated on M2
14 medium containing 0.5% DL lactate.

15
16 **Results :** In most human diets, resistant starch is
17 considered to be the most important energy source for
18 microbial growth in the large intestine (Topping, 2001).
19 The major amylolytic species in the human colon are
20 generally considered to be *Bacteroides* and
21 *Bifidobacterium* spp. (MacFarlane, 1986; Salyers, 1977).
22 Bifidobacteria produce acetate and lactate from
23 carbohydrate substrates, typically in the molar ratio of
24 3:2. Since the lactate utilisers isolated here either do
25 not utilise starch or utilised it weakly, as a growth
26 substrate in pure culture, it was of interest to co-
27 culture them with a starch-degrading *Bifidobacterium*
28 strain in order to establish whether they could remove
29 the lactate formed. The recently isolated, actively
30 amylolytic *B. adolescentis* strain L2-32 was used for
31 these experiments. As shown in Tables 3a, 3b and Fig. 2,
32 co-culture with any one of three lactate utilisers

1 tested, with starch as the growth substrate, resulted in
2 complete conversion of the L-lactate, and some of the
3 acetate, formed by *B. adolescentis* L2-32 into butyric
4 acid. This corresponded with greatly increased growth of
5 the lactate utilisers in the presence of the *B.*
6 *adolescentis* L2-32, as determined by selective plating.
7 Viable counts (cfu ml⁻¹) after 24 hours growth for L1-92,
8 SM 6/1 and L2-7 were, respectively, 2.4 x 10⁸, 1.0 x 10⁷
9 and 8.0 x 10⁶, in the absence of *B. adolescentis*, and 1.7
10 x 10⁹, 6.8 x 10⁸ and 5.4 x 10⁹, in the presence of *B.*
11 *adolescentis* L2-32. Growth of *B. adolescentis* L2-32 was
12 unaffected by co-culture (mean 4.3 x 10⁸ cfu ml⁻¹). There
13 may have been some contribution of starch hydrolysis
14 products that escape uptake by the *B. adolescentis* L2-32,
15 in addition to lactate and acetate, to the growth of the
16 lactate utilisers. This might account for the apparent
17 effectiveness of *E. hallii* SM 6/1 in co-culture, even
18 though this strain used rather little in pure culture
19 when supplied with lactate alone.

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1 Table 3a. Fermentation profiles for *Bifidobacterium*
 2 *adolescentis* L2-32 and three lactate utilisers when
 3 incubated alone or in co-culture for 24 hours at 37°C on
 4 modified YCFA medium (modified to contain 0.1% casitone)
 5 containing 0.2% soluble starch.

6

Culture/ co-culture	Formate	Acetate	Butyrate	Total Lactate	L-Lactate
L2-32	4.29±0.92	51.04±5.44	0	5.00±0.09	5.16±0.45
L1-92	0.01±0.01	34.99±0.93	1.57±0.26	0.40±0.69	0
SM 6/1	0	35.25±2.15	0.75±0.06	0.27±0.27	0
L2-7	0.04±0.06	35.70±0.44	0.83±0.02	0	0
L2-32+L1-92	4.29±0.04	44.82±1.13	7.62±0.66	0.61±0.53	0
L2-32+ SM 6/1	4.81±1.08	48.17±6.47	6.23±1.15	0	0
L2-32+L2-7	5.16±1.37	43.88±3.74	7.35±0.27	0.36±0.01	0

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1 Table 3b. Total viable counts (cfu per ml) of
2 *Bifidobacterium adolescentis* L2-32 and three lactate
3 utilisers following 24 hours at 37°C in monoculture and
4 co-culture. *Bifidobacterium adolescentis* L2-32 was
5 selected for on MRS + 0.25% propionate roll tubes and the
6 butyrate producing/lactate utilisers were selected for on
7 M2 + 0.5% lactate roll tubes following incubation for 24
8 hours at 37°C.

9

Culture / Co-culture	<i>B. adolescentis</i> L2-32	Butyrate producer / lactate utiliser
L2-32	3.8×10^8	
L1-92		2.4×10^8
S M6/1		1.0×10^7
L2-7		8.0×10^6
L2-32+L1-92	6.4×10^8	1.7×10^9
L2-32+SM 6/1	3.8×10^8	6.8×10^8
L2-32+L2-7	3.2×10^8	5.4×10^9

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1 Example 6: Time Course of Lactate Utilisation

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3 Time courses were followed in batch culture for growth on
4 glucose, lactate or glucose and lactate (Figs. 4, 5). *E.*
5 *hallii* L2-7 when grown with DL-lactate used all of the
6 added lactate together with some acetate, producing more
7 than 20 mM butyrate (Fig. 4). Less butyrate, but
8 significant formate, was produced during growth on
9 glucose, or on glucose plus lactate, and lactate
10 utilisation was almost abolished by the presence of
11 glucose. Hydrogen production in 24 hours was 12 $\mu\text{m ml}^{-1}$
12 for growth on glucose, 15.5 $\mu\text{mol ml}^{-1}$ for growth on
13 lactate and 10.9 $\mu\text{mol ml}^{-1}$ for growth on glucose plus
14 lactate. *A. caccae* L1-92 similarly produce larger
15 quantities of butyrate when grown on lactate compared
16 with growth on glucose, when formate was also a product.
17 This strain was able to use lactate once glucose had been
18 exhausted, following inoculation into glucose plus
19 lactate medium.

20

21 Strain SS2/1 is likely to represent a new species, since
22 its closest relative (95% identity in 16S rRNA sequence)
23 is the non-butyrate producing *Clostridium indolis*. This
24 strain was able to use D-, but not L-, lactate following
25 glucose exhaustion in lactate plus glucose medium (Fig.
26 5). Again formate was not a significant product when
27 lactate was the sole energy source but 4.7 $\mu\text{mol ml}^{-1}$
28 hydrogen was formed.

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1 **Summary**

2
3 A. caccae strain L1-92 was able to consume up to 30mM DL
4 lactate, along with 20-30 mM acetate during batch culture
5 incubation for 24 hours at 37°C with the production of
6 >20mM, and up to 45mM butyrate; this occurred also when
7 glucose was added as an alternative energy source (Table
8 1). Lactate or lactate plus glucose thus resulted in
9 very much higher production of butyrate than observed
10 with 23mM glucose alone, when only <15mM butyrate was
11 formed. Furthermore none of the 74 strains screened
12 previously by Barcenilla et al. (2000) produced more than
13 25mM butyrate when tested in M2GSC medium. Lactate
14 consumption is not a general characteristic of butyrate-
15 producers, and six of the strains screened in Table 1
16 failed to consume lactate in M2GSCL medium.

17
18 Six further strains that are highly active lactate
19 utilisers (defined for example as net consumption of at
20 least 10mM of lactate during growth to stationary phase
21 or for 24 hours in YCFALG or YCFAL medium at 37°C - see
22 Table 2a) were obtained following deliberate screening of
23 new human faecal isolates for lactate utilisation. At
24 least two of these (SL 6/1/1 and SM 6/1 - Tables 1, 2)
25 are related to *Eubacterium hallii*. (Table 2a), based on
26 determination of their 16S rDNA sequences. These
27 isolates again consume large quantities of lactate and
28 produce high levels of butyrate *in vitro*. With one
29 exception where considerable glucose repression occurred
30 (strain SL 6/1/1), significant lactate utilization
31 occurred in the presence of glucose (Table 2). Three
32 strains (Ss 2/1, Sr 1/1 and Ssc/2) showed preferential

1 utilization of D-lactate, whereas the two *E. hallii*-
2 related strains SM 6/1, SL 6/1/1 and *A. caccae* L1-92
3 utilise both isomers (Table 2b). The two stereoisomers
4 differ in their toxicity in the human body, with the D-
5 isomer being regarded as the more toxic (Chan et al.,
6 1994, Hove et al., 1995). The present invention thus
7 provides a means of utilising both D and L lactate
8 isomers or preferentially utilising D-lactate in
9 preference to L-lactate.

10
11 *A. caccae* and newly isolated bacteria related to *E.*
12 *hallii* and *C1. indolis* were shown to consume up to 30mM
13 DL, D or L lactate, along with 20-30 mM acetate during
14 batch culture incubation and convert this energy in to
15 production of at least 20mM, and up to 45mM butyrate.
16 Furthermore, these strains were shown to convert all of
17 the L-lactate produced by a starch-degrading strain of
18 *Bifidobacterium adolescentis* into butyrate when grown in
19 culture. This is the first documentation demonstrating
20 the conversion of lactate to butyrate by human colonic
21 bacteria, some of which are likely to be new species.

- 1 References
- 2
- 3 Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and
4 Lipman, D.J. (1990) Basic local alignment search tool. J.
5 Mol. Biol. 215, 403-410.
- 6
- 7 Archer, S.Y., Meng, S.F., Sheh, A. and Hodin, R.A.
8 (1998). p21 (WAF1) is required for butyrate mediated
9 growth inhibition of human colon cancer cells. Proc.
10 Natl. Acad. Sci. USA, 95, 6791-6796.
- 11
- 12 Atlas, R.M. (1997). Handbook of microbiological media
13 (2nd edition). Ed. L.C. Park. CRC Press, Cleveland, Ohio.
- 14
- 15 Barcenilla, A., Pryde, S.E., Martin, J.C., Duncan, S.H.,
16 Stewart, C.S., Henderson, C. and Flint, H.J. (2000).
17 Phylogenetic relationships of dominant butyrate producing
18 bacteria from the human gut. Appl. Environ. Microbiol.,
19 66, 1654-161.
- 20
- 21 Bourriaud, C., Akoka, S., Goupry, S., Robins, R.,
22 Cherbut, C. and Michel, C. (2002). Butyrate production
23 from lactate by human colonic microflora. Reprod. Nutr.
24 Develop., 42, (Suppl. 1). S55.
- 25
- 26 Brosius, J., Palmer, M.L., Kennedy, P.J. and Noller, H.F.
27 (1978) Complete nucleotide sequence of a 16S ribosomal
28 RNA gene from Escherichia coli. Proc. Natl. Acad. Sci.
29 75, 480-4805.
- 30

- 1 Byrant, M.P. (1972) Commentary on the Hungate technique
2 for cultivation of anaerobic bacteria. Am. J. Clin.
3 Nutr. 25, 1324-1328.
- 4
- 5 Chan, L., Slater, J., Hasbargen, J., Herndon, D.N.,
6 Veech, R.L. and Wolf. S. (1994). Neurocardiac toxicity of
7 racemic D, L-lactate fluids. Integr. Physiol. Behav.
8 Sci., 29, 383-394.
- 9
- 10 Collins, M.D. and Gibson, G.R. (1999). Probiotics,
11 prebiotics, and symbiotics: approaches for modulating the
12 microbial ecology of the gut. Am. J. Clin. Nutr., 69
13 (suppl), 1052S-1057S.
- 14
- 15 Counotte, G.H.M., Prins, R.A., Janssen, R.H.A.M., DeBie,
16 M.J.A. (1981). Role of *Megasphaera elsdenii* in the
17 fermentation of DL-[2-¹³C] lactate in the rumen of dairy
18 cattle. Appl. Environ. Microbiol. 42: 649-655.
- 19
- 20 Csordas, A. (1996). Butyrate, aspirin, and colorectal
21 cancer. Europ. J. Cancer Prevent., 5, 221-231.
- 22 Day, A.S. and Abbott, G.D. (1999). D-lactic acidosis in
23 short bowel syndrome. New Zealand Med. J. 112: 277-278.
- 24
- 25 Duncan, S.H., Hold, G.L., Barcenilla, A., Stewart, C.S.
26 and Flint, H.J. (2002). *Roseburia intestinalis* sp. nov.,
27 a novel saccharolytic, butyrate producing bacterium from
28 human faeces. Int. J. System. Evol. Microbiol., 52, 1-6.
- 29
- 30 Gilmour, M., Flint, H.J. and Mitchell, W.J. (1994).
31 Multiple lactate-dehydrogenase activities of the rumen

- 1 bacterium *Selenomonas ruminantium*. *Microbiol.*, 1440,
2 2077-2084.
3
4 Hold, G.L., Pryde, S.E., Russell, V.J., Furrie, E. and
5 Flint, H.J. (2002). The assessment of microbial diversity
6 in human colonic samples by 16S rDNA sequence analysis.
7 *FEMS Microbiol. Ecol.*, 39, 33-39.
8
9 Hove, H., Nordgraard-Andersen, I. and Mortensen, B.
10 (1994). Faecal DL-lactate concentration in 100
11 gastrointestinal patients. *Scand. J. Gastroenterol.*, 29,
12 255-259.
13
14 Hove, H., Holtug, K., Jeppesen, P.B. and Mortensen, P.B.
15 (1995). Butyrate absorption and lactate secretion in
16 ulcerative colitis. *Dis. Colon Rect.*, 38, 519-525.
17
18 Inan, M.S., Rasoulpour, R.J., Yin, L., Hubbard, A.K.,
19 Rosenberg, D.W. and Giardina, C. (2000). The luminal
20 short-chain fatty acid butyrate modulates NF kappa B
21 activity in a human colonic epithelial cell line.
22 *Gastroenterol.* 118: 724-734.
23
24 Kanauchi, O., Fujiyama, Y., Mitsuyama, K., Araki, Y.,
25 Ishii, T., Nakamura, T., Hitomi, Y., Agata, K., Saiki,
26 T., Andoh, A., Toyonaga, A., and Bamba, T. (1999).
27 Increased growth of *Bifidobacterium* and *Eubacterium* by
28 germinated barley foodstuff, accompanied by enhanced
29 butyrate production in healthy volunteers. *Int. J. Mol.*
30 *Med.*, 3, 175-179.
31

- 1 Kung, L.M. and Hession, A.O. (1995). Preventing in vitro
2 lactate accumulation in ruminal fermentations by
3 inoculation *Megasphaera elsdenii*. J. Anim. Sci., 73, 250-
4 256.
- 5
- 6 MacFarlane, G.T. and Englyst, H.N. (1986) Starch
7 utilisation by the human large intestinal microflora. J.
8 Appl. Bacteriol. 60, 195-201.
- 9
- 10 Nocek, J.E. (1997). Bovine acidosis: implications on
11 laminitis. J. Dairy Sci., 80, 1005-1028.
- 12
- 13 Ouwerkerk, D., Klieve, A.V. and Forster, R.J. (2002).
14 Enumeration of *Megasphaera elsdenii* in rumen contents by
15 real-time Taq nuclease assay. J. Appl. Microbiol., 92,
16 753-758.
- 17
- 18 Pryde, S.E., Duncan, S.H., Hold, G.L., Stewart, C.S. and
19 Flint, H.J. (2002). The microbiology of butyrate
20 formation in the human colon. FEMS Microbiol. Letts.,
21 217, 133-139.
- 22
- 23 Russell, J.B. and Rychlik, J.L. (2001). Factors that
24 alter rumen microbial ecology. Science, 292, 1119-1122.
- 25
- 26 Salyers, A.A., West, S.E.H., Vercellotti, J.R. and
27 Wilkins, T.D. (1977) Fermentation of mucus and plant
28 polysaccharides by anaerobic bacteria from the human
29 colon. Appl. Environ. Microbiol. 34, 529-533.
- 30
- 31 Schwiertz, A., Hold, G.L., Duncan, S.H., Gruhl, B.,
32 Collin, M.D., Lawson, P.A., Flint, H.J. and Blaut, M.

1 (2002). *Anaerostipes caccae* gen. nov., sp. nov., a new
2 saccharolytic, acetate-utilising, butyrate-producing
3 bacterium from human faeces. *Syst. Appl. Microbiol.*, 25,
4 46-51.

5

6 Suau, A., Bonnet, R., Sutren, M., Godon, J.J., Gibson,
7 G.R., Collins, M.D. and Dore, J. (1999). Direct analysis
8 of genes encoding 16S rRNA from complex communities
9 reveals many novel molecular species within the human
10 gut. *Appl. Environ. Microbiol.*, 65, 4799-4807.

11

12 Topping, D.L. and Clifton P.M. (2001) Short chain fatty
13 acids and human colonic function : roles of resistant
14 starch and nonstarch polysaccharides. *Physiol. Revs.* 81,
15 1031-1064.

16

17 Trinder, P. (1969) Determination of glucose in blood
18 using glucose oxidase with an alternative oxygen
19 acceptor. *Ann. Clin. Biochem.* 6, 24/27.

20

21 Ushida, K., Hashizume, K., Tsukahara, T., Yamada, K. and
22 Koyama, K. (2002). *Megasphaera elsdenii* JCM 1772^T
23 regulates hyper lactate production in the rat large
24 intestine. *Reprod. Nutr. Develop.*, 42 (Suppl. 1) S56-S57.

25

26 Weisburg, M.J., Miller, T.L., Yerry, S., Zhang, Y.C.
27 Bank, S. and Weaver, G.A. (1991) Changes of fermentation
28 pathways of faecal microbial communities associated with
29 a drug treatment that increases dietary starch in the
30 human colon. *Appl. Environ. Microbiol.* 65, 2807-2812.

31

- 1 Wiryawan, K.G. and Brooker, J.D. (1995). Probiotic
2 control of lactate accumulation in acutely grain-fed
3 sheep. Austral. J. Agric. Res., 46, 1555-1568.
4

1 CLAIMS

2

3 1. A method of selecting a strain of lactic acid-
4 utilising bacteria, which method comprises the
5 steps of:

6

- 7 a) providing a bacterial culture from a human
8 faecal sample;
9 b) selecting a single colony of bacteria;
10 c) growing said colony in a suitable medium
11 containing lactic acid; and
12 d) selecting a strain of bacteria consuming
13 relatively large amounts of lactic acid,
14
15 all of the above steps being conducted under
16 anaerobic conditions.

17

18 2. The method as claimed in Claim 1 wherein at
19 least 10mM of lactic acid is consumed during
20 growth into the stationary phase per 24 hours
21 at 37°C in YCFALG or YCFAL medium.

22

23 3. The method as claimed in either one of Claims 1
24 and 2 wherein said method comprises the
25 additional step of:

26

27 e) selecting a strain of bacteria producing
28 relatively large quantities of butyric
29 acid.

30

31 4. The method as claimed in Claim 3 wherein at
32 least 10mM butyric acid is produced during

- 1 bacterial growth into the stationary phase per
2 24 hours at 37°C in YCFALG or YCFAL medium.
3
- 4 5. The method as claimed in any one of Claims 1 to
5 4 wherein said lactic acid is a mixture of D
6 and L isomers of lactic acid.
7
- 8 6. *Anaerostipes caccae* strain L1-92 deposited at
9 NCIMB under No. 13801^T.
10
- 11 7. *Clostridium indolis* bacterial strain Ss2/1
12 deposited at NCIMB under No. 41156.
13
- 14 8. *Eubacterium hallii* strain SM 6/1 deposited at
15 NCIMB under No. 41155.
16
- 17 9. A lactic acid utilising bacterium having a 16S
18 rRNA gene sequence with at least 95% homology
19 to one of the sequences shown in Fig. 1.
20
- 21 10. A bacterial strain as claimed in any one of
22 Claims 6 to 9 for use as a medicament to treat
23 lactic acid induced disorders or as a
24 foodstuff.
25
- 26 11. A bacterial strain as claimed in Claim 10 for
27 use as a medicament to treat lactic-acidosis,
28 short bowel syndrome or inflammatory bowel
29 disease.
30
- 31 12. A method to promote butyric acid formation in
32 the intestine of a mammal, said method

1 comprising the administration of a
2 therapeutically effective dose of at least one
3 of the strains of bacteria as claimed in any
4 one of Claims 6 to 9.

5

6 13. The method of Claim 12 wherein said bacteria is
7 administered as a foodstuff or as a
8 suppository.

9

10 14. A method for treating a disease associated with
11 a high dosage of lactic acid, which method
12 comprises the administration of a
13 therapeutically effective dose of at least one
14 strain of live lactic acid utilising bacteria
15 as claimed in any one of Claims 6 to 9.

16

17 15. The method of Claim 14 wherein said disease is
18 lactic-acidosis, short bowel syndrome or
19 inflammatory bowel disease.

20

21 16. The method of either one of Claims 14 and 15
22 wherein said bacteria is *Anaerostipes caccae*.

23

24 17. A prophylactic method to reduce the incidence
25 or severity of colorectal cancer or colitis in
26 mammals caused in part by high lactic acid and
27 low butyric acid concentrations, which method
28 comprises the administration of a
29 therapeutically effective dose of at least one
30 strain of live lactic acid utilising bacteria
31 as claimed in any one of Claims 6 to 9.

32

1 18. The method of Claim 17 wherein said bacteria is
2 *Anaerostipes caccae*.

3

4 19. A probiotic composition comprising a live
5 bacterial strain as claimed in any one of
6 Claims 6 to 9, in combination with live lactic
7 acid producing bacteria.

8

9 20. The composition as claimed in Claim 19 wherein
10 said lactate acid producing bacteria is
11 *Lactobacillus spp*, *Bifidobacterium spp* or a
12 mixture thereof.

13

14 21. The method of either one of Claims 19 and 20
15 wherein said bacteria is *Anaerostipes caccae*.

16

17 22. The composition as claimed in any one of Claims
18 19 to 21 further containing other additives or
19 growth enhancing supplements.

1 **Figure 1**

2

3 Sequence information for five of the lactate utilising
4 strains.

5

6 **S D6 1L/1**

7 GATGAACGCTGGCGCGTGCCTAACACTGCAAGTCGAACGAAGCACCTTACCTGATTCTCGGATGAA
8 GGTCTGGTACTGAGTGGCGGACGGGTGAGTAACGCGTGGTAACCTGCCCTGTACAGGGGGATAACA
9 GTTGGAAACGGCTGCTAATACCGCATAAGCGCACGAGAGGACATCCTCTTGTGAAAAACTCCGGTG
10 GTACAGGATGGGCCCGTCTGATTAGCTGGTGGCAGGGTAACGGCCTACCAAGGCACGATCAGTA
11 GCCGGTCTGAGAGGATGAACGGCCACATTGAACTGAGACACGGTCCAACTCATAACGGGAGGCAGCAG
12 TGGGAATATTGCACAATGGGGAAACCTGATGCAGCAACGCCCGTGAGTGAAGAAGTATTTCGGT
13 ATGTAAAGCTCTATCAGCAGGGAAAGATAATGACGGTACCTGACTAAGAAGCTCCGGCTAAATACGTGC
14 CAGCAGCCCGGTAATACGTATGGAGCAAGCGTTATCCGGATTACTGGGTGAAAGGGTAGCTAG
15 GGCAGTGCAGTCAGATGTGAAAGGCCGGCTCAACCCCGAGCTGCATTGAAACTGCATAGCTAG
16 AGTACAGGAGAGGCAGGCCGAATTCTAGTGTAGCGGTGAAATGCGTAGATATTAGGAGGAACACCAG
17 TGGCGAAGGCCGCTGCTGGACTGTTACTGACACTGAGGCACGAAAGCGTGGGAGCAAACAGGATTA
18 GATACCTGGTAGTCCACGCCGTAAACGATGAATACTAGGTGTCGGGCCGTAGGCTTCGGTGC
19 TCGCAAACCGCAGTAAGTATTCCACCTGGG
20 GAGTACGTTGCAAGAATGAAACTCAAAGGAATTGACGGGACCCGCACAAGCGTGGAGCATG
21 TGGTTAATTGCAAGCAACCGCAAGAACCTTACCAAGGTCTTGACATCCTCTGACCACCTCGTA
22 ATGGGAGTCTCCTTCGGACAGAAGAGACAGGTGGTGATGGTTGTCGTCACTCGTGT
23 AGATGTTGGGTTAAGTCCCACGAGCGAACCCCTATCTCAGTAGCCAGCAGGTAAAGGCTG
24 GGCACCTGGAGAGACTGCCAGGGATAACCTGGAGGAAGGTGGGACGACGTCAAATCATCATG
25 CCCCTATGATCTGGCGACACACGTGCTACAATGGCGGTACAAAGTGAGGCGAACCTCGCAG
26 GGGGAGCAAACCAACAAAAGGCCGTCCAGTCGGACTGTAGTCTGCAACCCGACTACACGAAG
27 CTGGAATCGCTAGTAATCGCAATCAGAATGTCGCGGTGAATACGTTCCCGGTCTGTACACA
28 CCGCCCGTACACCATGGAGTCGGAAATGCCGAAGCCAGTGACCCAACCATAATGGAGGGAGC
29 TGTCGAAGGTGGAGCCGGTAACTGGGGTG
30

31 **SM 6/1**

32 GATGAACGCTGGCGCGTGCCTAACACATGCAAGTCGAACGAAGCACCTTACGAGATTCTCGGATG
33 TCGTTGGTACTGAGTGGCGGACGGGTGAGTAACGCGTGGTAACCTGCCCTGTACAGGGGGATAAC
34 AGCTGGAAACGGCTGCTAACCCGATAAGCGCACGAGGAGACATCCTCTAGTGTGAAAAACTCCGGT
35 GGTACAGGATGGGCCCGTCTGATTAGCTGGTGGCAGGGTAACGGCCTACCAAGGCACGATCAGT
36 AGCCGGTCTGAGAGGATGAACGGCCACATTGAACTGAGACACGGTCAAACCTCCTACGGGAGGCAGC
37 AGTGGGAATATTGCACAATGGGGAAACCTGATGCAGCAACGCCCGTGAGTGAAGAAGTATTTCG
38 GTATGTAAGCTCTATCAGCAGGGAAAGATAATGACGGTACCTGACTAAGAAGCTCCGGCTAAATACGT

1 GCCAGCAGCCGCGGTAAATAGATATGGAGCAAGCGTTATCCGGATTACTGGGTGTAAAGGGTGCCTAG
2 GTGGCAGTGCAAGTCAGATGTGAAAGGCCGGGCTCAACCCGGAGCTGCATTTGAAACTGCWYRGCT
3 AGAGTACAGGAGAGGCAGGCAGGAATTCTAGTGTAGCGGTGAAATCGTAGATATTAGGAGGAACACC
4 AGTGGCGAAGGCCGCTGCTGGACTGTTACTGACACTGAGGCACGAAAGCGTGGGAGCAAACAGGAT
5 TAGATACCCTGGTAGTCCACGCCGTAACCGATGAATACTAGGTGTGGGCCGTATAGGCTCCGGTGC
6 CGCCGCTAACGCACTAACGAGTATTCCACCTGGGAGTACGTCGAAGAATGAAACTCAAAGGAATTGAC
7 GGGGACCCGACAAGCGGTGGAGCATGTGGTTAATCGAAGCAACCGAAGAACCTTACCAAGGTCTT
8 GACATCCTCTGACCGCACCTTAATCGGTGCTTCCTTCGGACAGAAGAGACAGGTGGTGCATGGTT
9 GTCGTCAGCTCGTGTGAGATGTTGGGTTAAGTCCCACGAGCGAACCCCTATCTTCAGTAGC
10 CAGCAGGTAAGGCTGGGCACTCTGGAGAGACTGCCAGGGATAACCTGGAGGAAGGTGGGACGACGTC
11 AAATCATCATGCCCTTATGATCTGGCGACACACGTGCTACAATGGCGGTACAGAGTGAGGCGAAC
12 CCGCGAGGGGGAGCAAACCAACAAAAAGCCGTCCCAGThCGGACTGTAGTCTGCAACCCGACTACACA
13 GAAGCTGGAATCGCTAGTAATCGCAATCAGAATGTCGCGGTGAATACGTTCCGGGTCTTGTACACA
14 CCGCCCGTCACACCATGGGAGTCGGAAATGCCGAAGCCAGTGACCCAACCTTATGAAGGAAGCCnG
15 TCCAAGGTTGAACCCGTTAACTGGGnnTT
16
17 **Ss3/4**

18 GAGTTTGATCCTGGCTCAGGATGAACGCTGGCGCGTGCCTAACACATGCAAGTCGAACGAGGT
19 ATATTGAATTGAAGTTTCGGATGGATTCAATGATAACCGAGTGGCGGACGGGTGAGTAACGCGTGGG
20 TAACCTGCCTCATACAGGGGATAACGGTTAGAAATGACTGCTAACCGCATAAGCCACA
21 GTACCGCATGGTACGGTGTAAAAACTCCGGTGGTATGAGATGGACCCCGCTGATTAGCTAG
22 TTGGTGGGTAACGGCCCACCAAGGCACGATCAGTAGCCGACCTGAGAGGGTGACGGCCACA
23 TTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTGGGGATATTGCACAATGGAG
24 GAAACTCTGATGCAGCGACGCCGTGAGTGAAGAAGTATTCCGTATGTAAGCTCTATCAGC
25 AGGGAAAGAAAATGACGGTACCTGACTAAGAAGCCCCGCTAACTACGTGCCAGCAGCCGGTA
26 ATACGTAGGGGCAAGCGTTATCCGGATTACTGGGTGAAAGGGAGCGTAGACGGCGACGCAA
27 GTCTGAAGTGAATAACCCGGCTCAACCTGGAACTGCTTGGAAACTGTGTTGCTAGAGTGCT
28 GGAGAGGTAAGCGGAATTCTAGTGTAGCGGTGAAATCGTAGATATTAGGAAGAACACCAGTG
29 GCGAAGGCGGCTTACTGGACAGTAACTGACGTTGAGGCTCGAAAGCGTGGGAGCAAACAGGAT
30 TAGATACCCTGGTAGTCCACGCCGTAAACGATGAATACTAGGTGTTGGTGAGCAAAGCTCATCG
31 GTGCCGCCAAACGCAATAAGTATTCCACCTGGGAGTACGTCGCAAGAATGAAACTCAAAG
32 GAATTGACGGGACCCGCACAAGCGGTGGAGCATGTGGTTAATCGAAGCAACCGAAGAAC
33 CTTACCAAATCTGACATCCCTCTGAAAARYCYTTAATCGGRTCCCTCCTCGGGACAGAGGT
34 GACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTGAGATGTTGGGTTAAGTCCCACGAG
35 CGCAACCCCTATTGTCAGTAGCCAGCAGGTGAAGCTGGGACTCTGATGAGACTGCCAGGGATA
36 ACCTGGAGGAAGGTGGGATGACGTCAAATCATCATGCCCTTATGATTGTTGGCTACACACGTG
37 CTACAATGGCGTAAACAAAGAGAAGCGAGCCTGCGAGGGGAGCAAATCTCAAAATAACGTCT
38 CAGTTCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAATCGCAGATCAG

1 AATGCTGCCGTGAATACGTTCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGTCGGAA
2 ATGCCCGAAGCCAGTGAACCCAATGCGAAAGCAGGGAGCTGTCGAAGGCAGGTCTGATAACTGGGTG
3

4 **Ss2/1 and Ssc/2**

5 AGAGTTGATCCTGGCTCAGGATGAACGCTGGCGCGTGCTTAACACATGCAAGTCGAACGAAA
6 CACCTTATTGATTTCTCGGAACTGAAGATTGGTGATTGAGTGGCGGACGGGTGAGTAACG
7 CGTGGGTAAACCTGCCCTGTACAGGGGATAAACAGTCAGAAATGACTGCTAATACCGCATAAGAC
8 CACAGCACCGCATGGTCAGGGTAAAAACTCCGGTGGTACAGGATGGACCCCGTCTGATTAG
9 CTGGTTGGTGAGGTAACGGCTACCAAGGCACGATCAGTAGCCGGTTGAGAGAGTGAACGGC
10 CACATTGGGACTGAGACACGGCCAAACTCCTACGGGAGGCAGCAGTGGGAATATTGCACAAT
11 GGGGAAACCTGATGCAGCGACGCCCGTGAGTGAAGAAGTATCTCGGTATGTAAGCTCTAT
12 CAGCAGGGAAGAAAATGACGGTACCTGACTAAGAAGCCCCGGCTAACTACGTGCCAGCAGCCGC
13 GGTAAACGTAGGGGCAAGCGTTATCCGAATTACTGGGTGTAAGGGTGCCTAGGTGGTATG
14 GCAAGTCAGAAGTGAAAACCCAGGGCTTAACTCTGGGACTGCTTTGAAACTGTCAGACTGGAG
15 TGCAGGAGAGGTAAGCGGAATTCTTAGTGTAGCGGTGAAATGCGTAGATATTAGGAGGAACATC
16 AGTGGCGAAGGCGGTTACTGGACTGAAACTGACACTGAGGCACGAAAGCGTGGGAGCAAACA
17 GGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATACTAGGTGTCGGGCCGTAGAGGC
18 TTCGGTGCCGCAGCCAACGCAGTAAGTATTCCACCTGGGAGTACGTTCGCAAGAATGAACTCA
19 AAGGAATTGACGGGACCCGCACAAGCGGTGGAGCATGTGGTTAATCGAAGCAACCGAAGA
20 ACCTTACCTGGCTTGACATCCTCTGACCGGTCTTAACCGGACTTTCTTCGGACAGGAG
21 TGACAGGTGGTCATGGTTGTCGTCAGCTCGTGTGAGATGTTGGGTTAAGTCCCACCGA
22 GCGCAACCCCTATCTTAGTAGCCAGCATATAAGGTGGGACTCTAGAGAGACTGCCAGGGATA
23 ACCTGGAGGAAGGTGGGACGACGTCAAATCATCATGCCCTTATGACCAGGGCTACACACGTG
24 CTACAATGGCGTAAACAGAGGGAAAGCAGCCTCGTGAGAGTGAGCAAATCCAAAAATAACGTCT
25 CAGTTCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAATCGCAATCAG
26 AATGTCGCGGTGAATACGTTCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGTCAGTA
27 ACGCCCGAAGTCAGTGACCCAACCGTAAGGAGGAGCTGCCGAAGCGGGACCGATAACTGGGTG
28 AAGTCGTAACCAGGTAGCCGT
29

30 W = A or T

31 Y = T or C

32 R = G or A

33 N = Unknown

34

Co-culture on starch

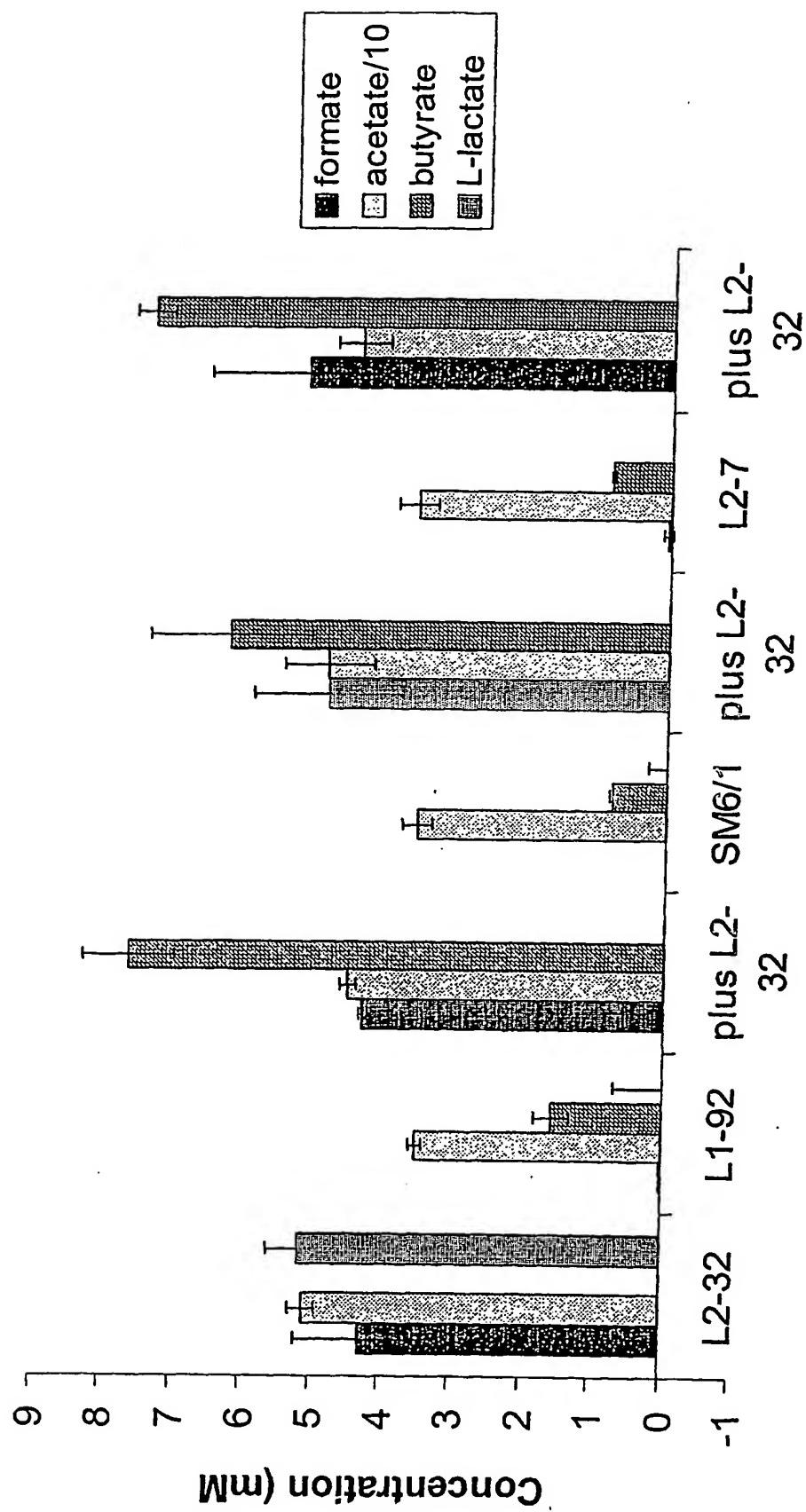


Fig. 2

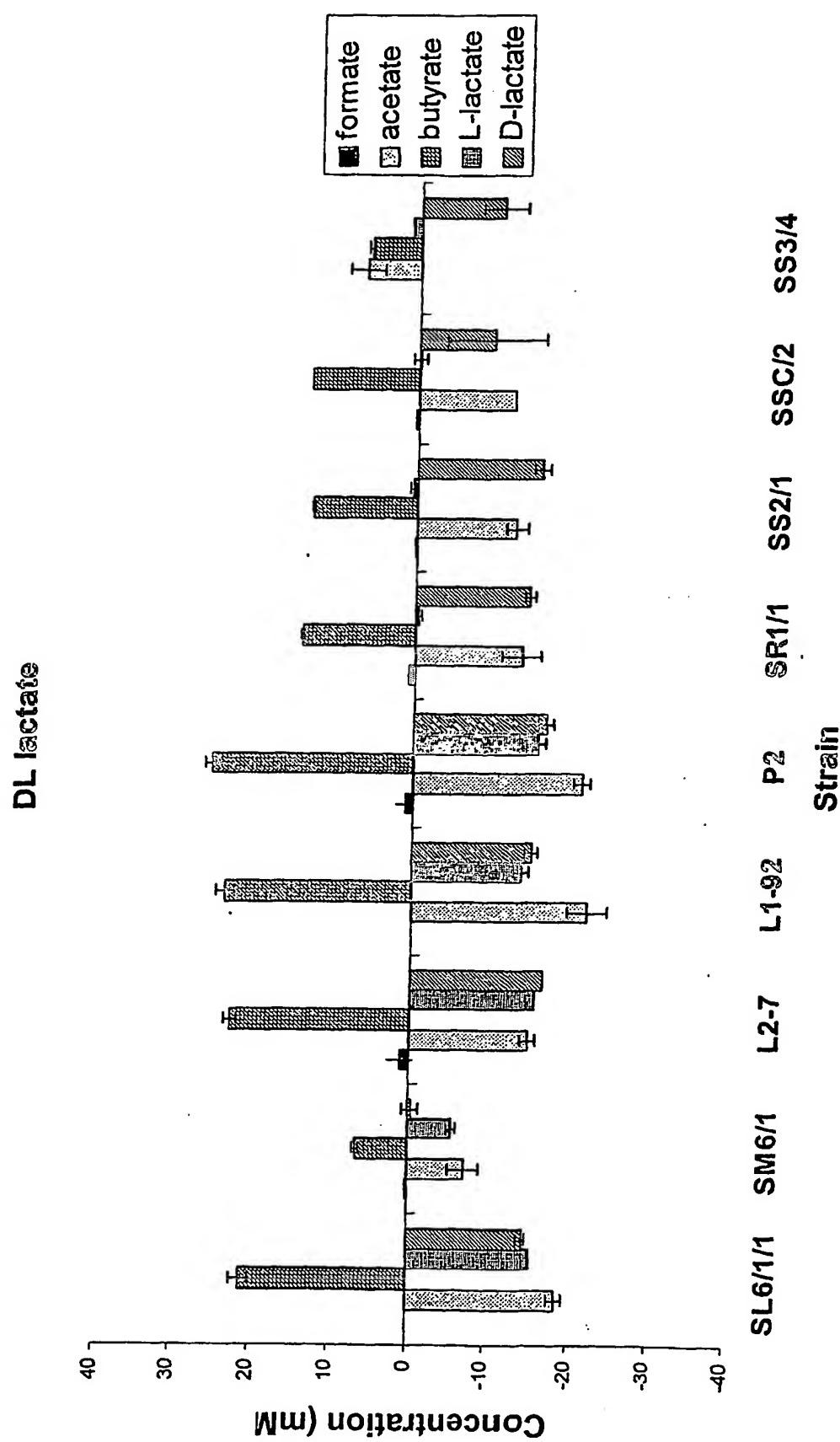
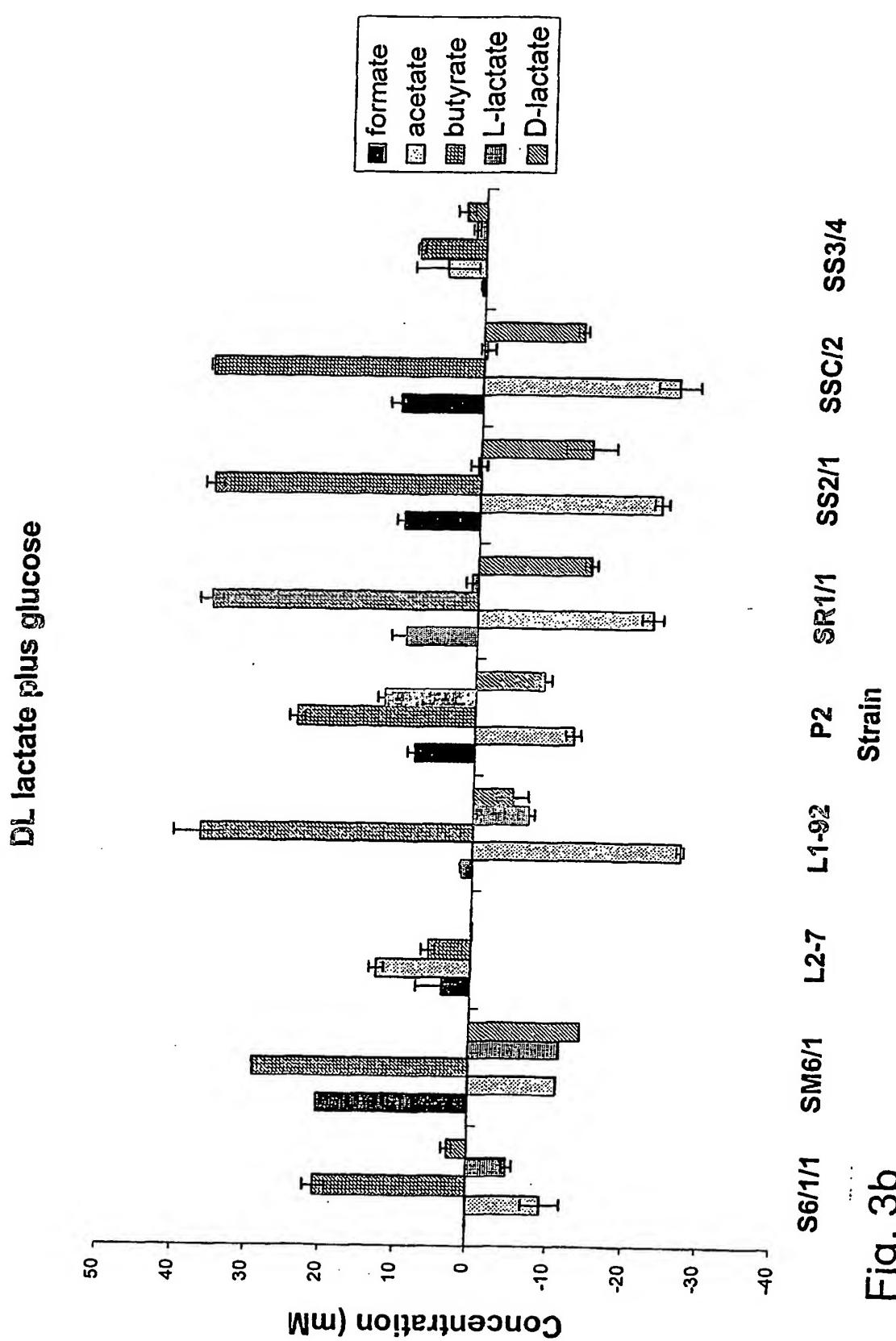


Fig. 3a

**Fig. 3b**

No addition

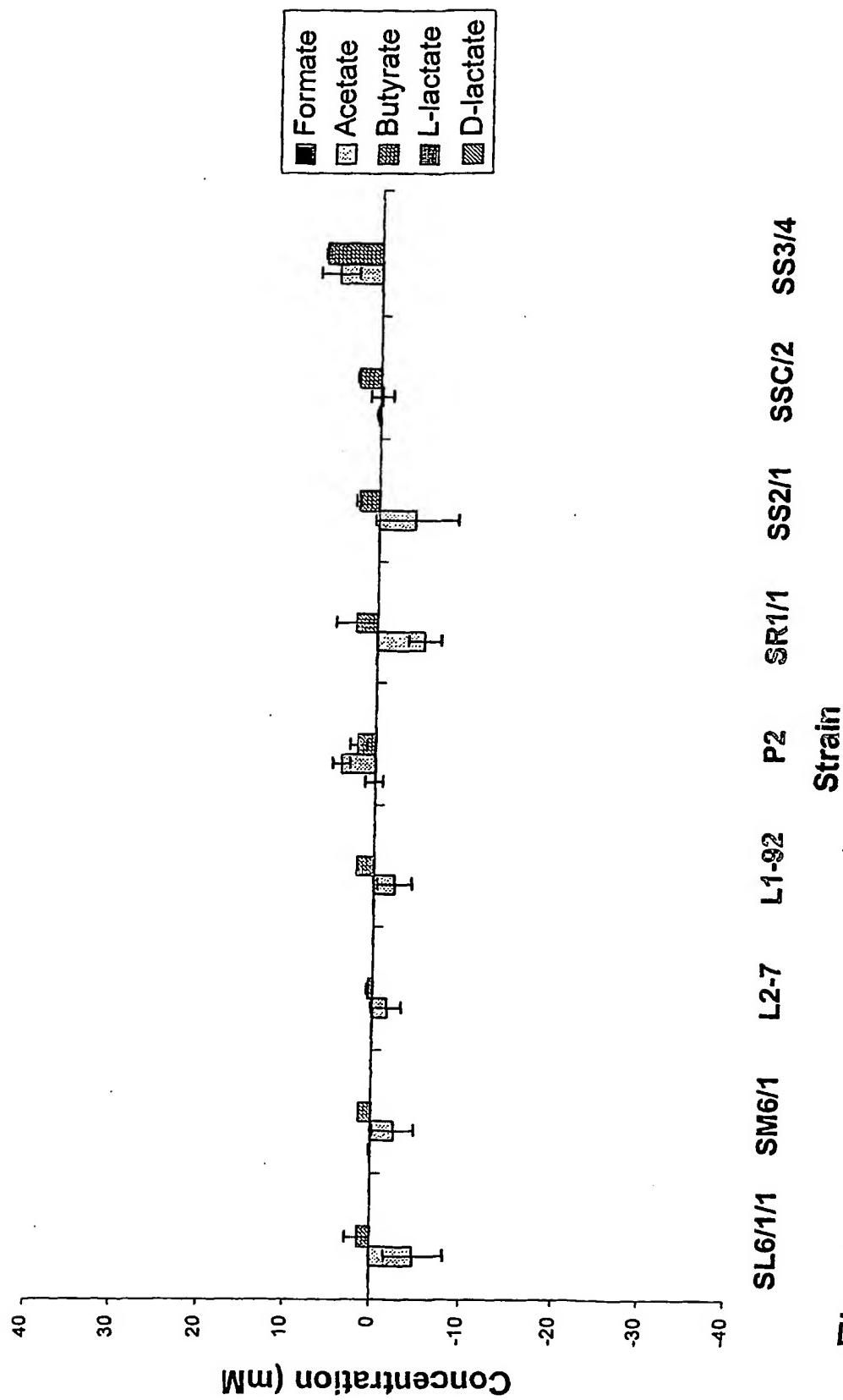


Fig. 3c

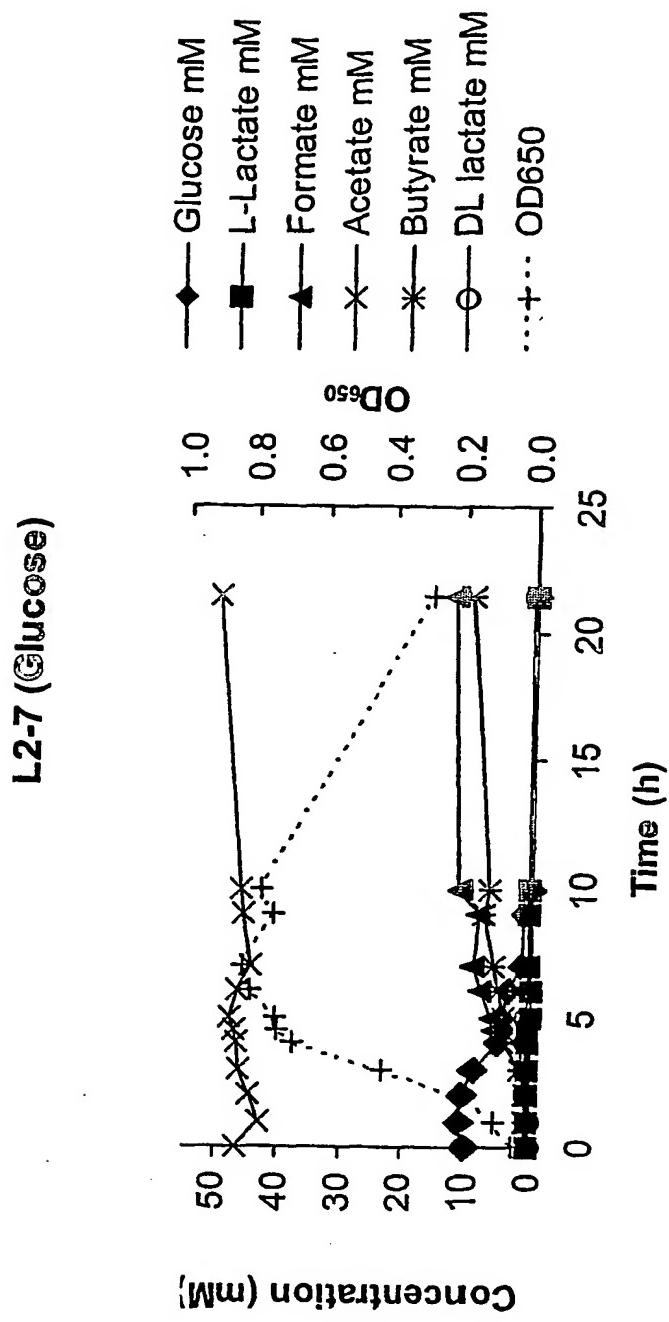


Fig. 4

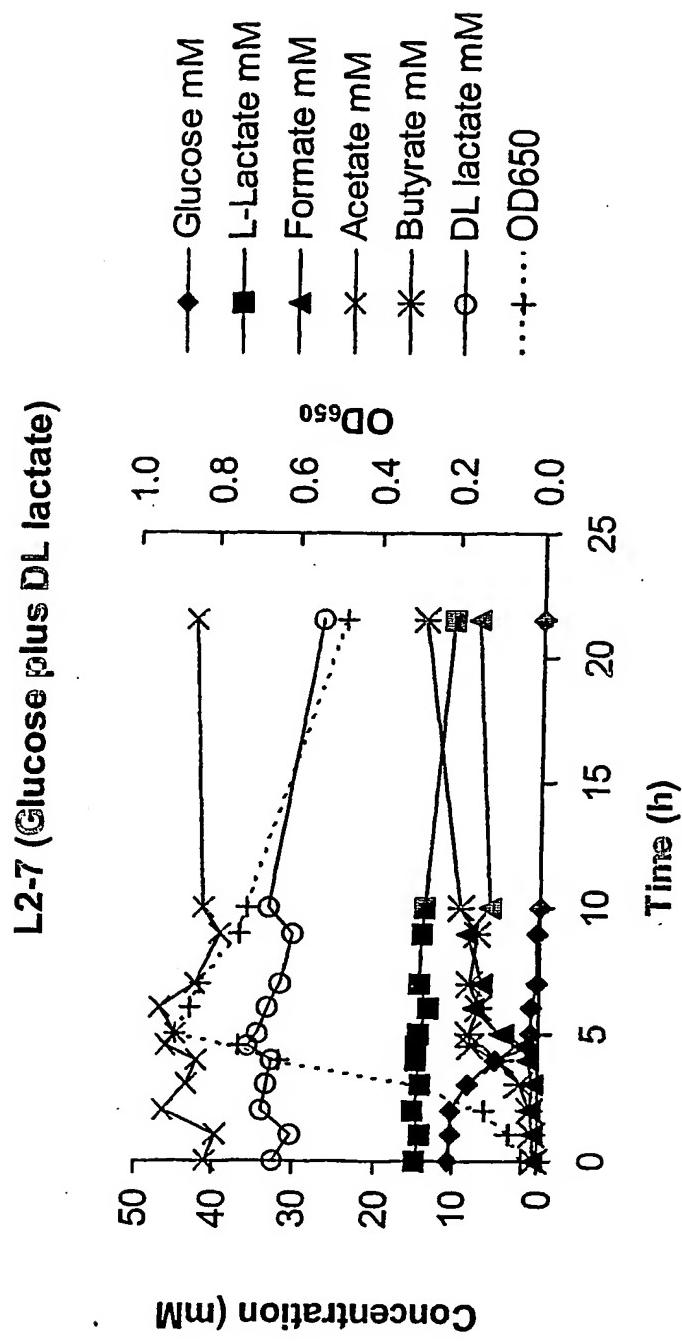


Fig. 4 continued

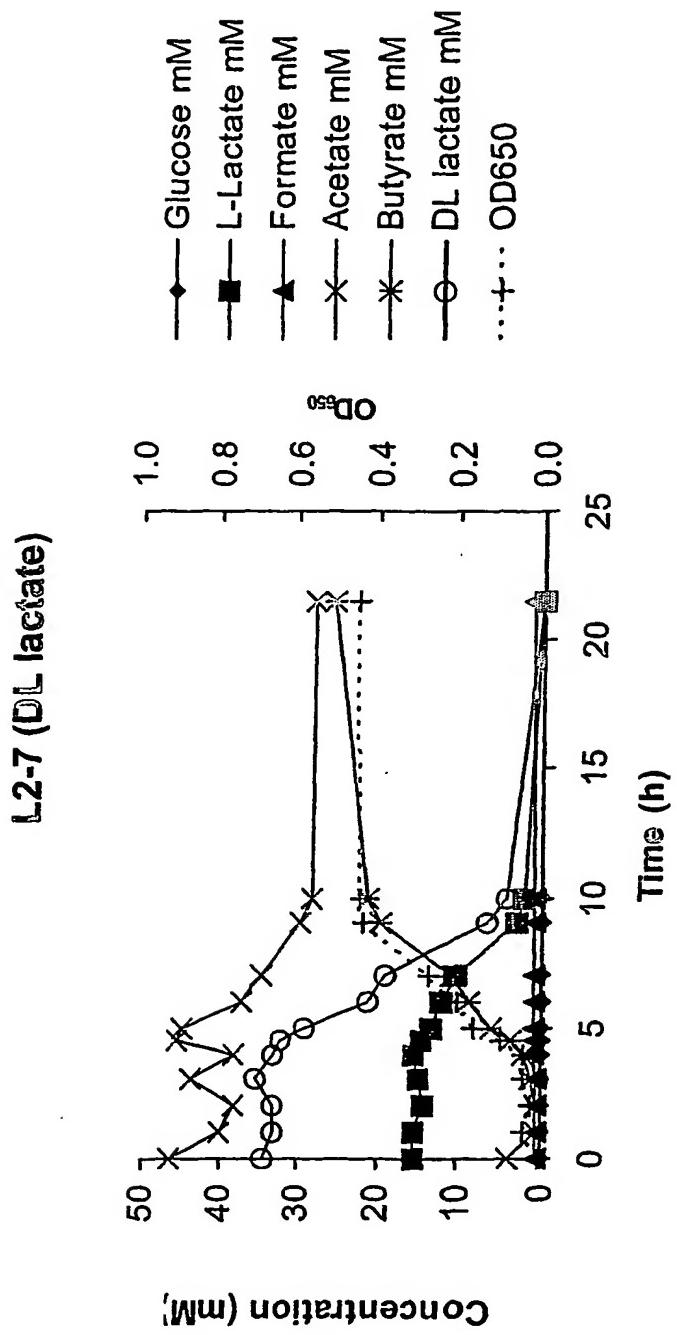


Fig. 4 continued

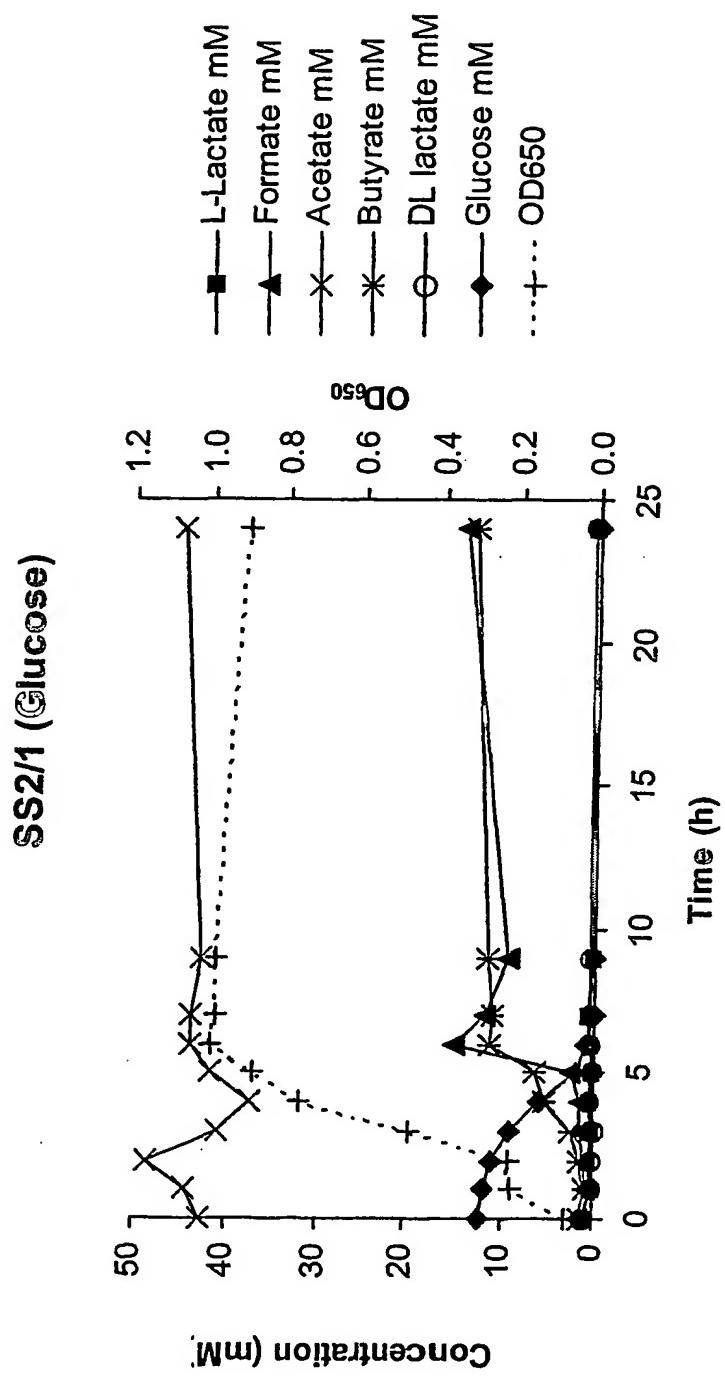


Fig. 5

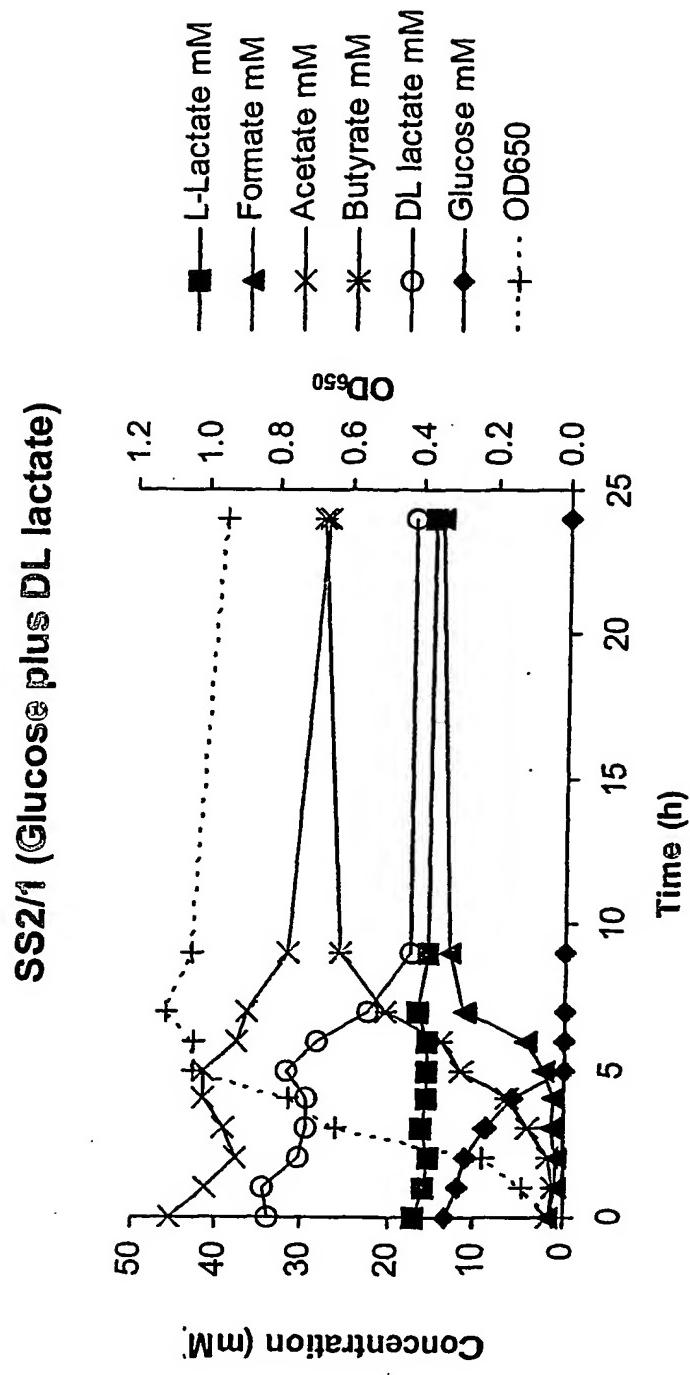


Fig. 5 continued

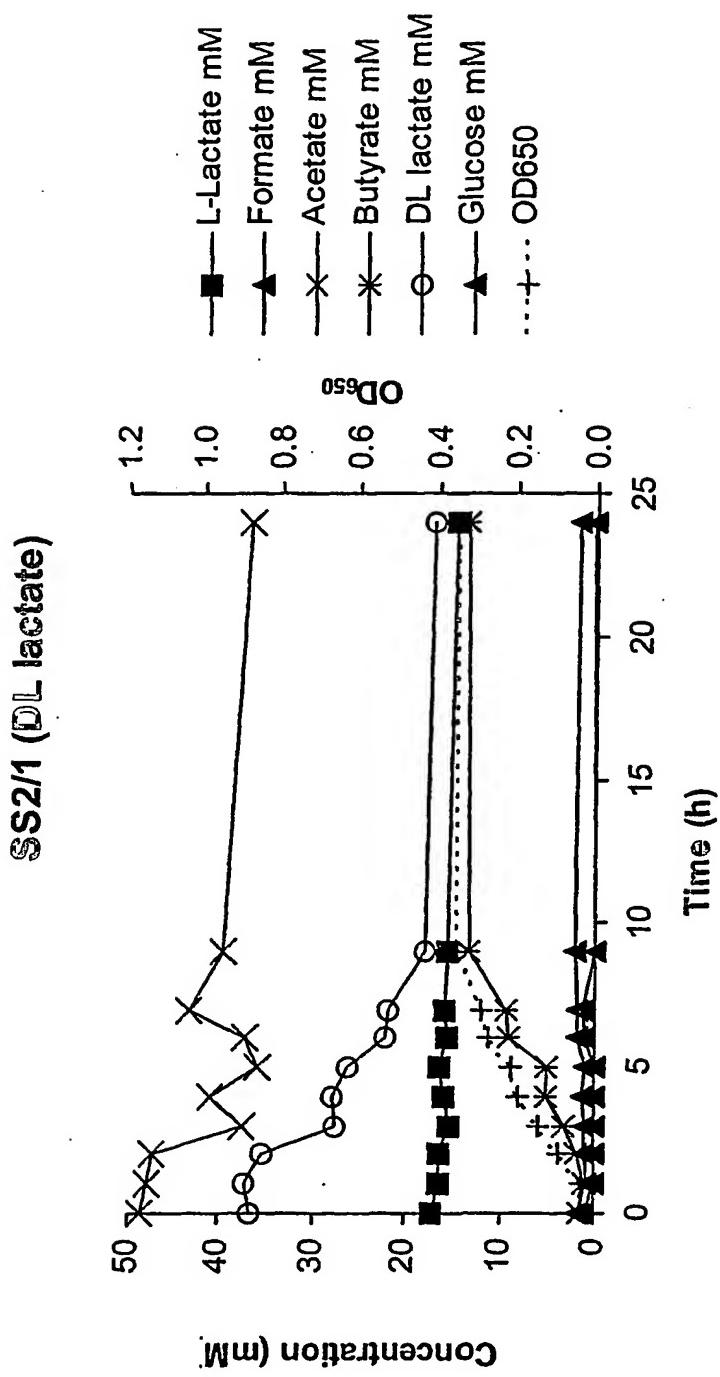


Fig. 5 continued